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Autoimmunity in the immune privileged eye: pathogenic and regulatory T cells

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Abstract Experimental autoimmune uveitis (EAU) in animals serves as a model of human uveitis. EAU can be induced in mice by immunization with the retinal antigen interphotoreceptor retinoid binding protein (IRBP) in complete Freund's adjuvant (CFA) or by IRBP-pulsed mature dendritic cells, and can be driven either by a Th17 or a Th1 effector response, depending on the model. The direction of the response is affected by conditions present during the exposure to antigen, including the quality/quantity of innate receptor stimulation and/or type of APC. IL-17 and IFN-γ production by innate cells such as NKT may also affect the disease process. If exposure to antigen is via a hydrodynamic DNA vaccination with an IRBP-encoding plasmid, the response is directed to a regulatory phenotype, and disease is ameliorated or prevented. Our data shed light on effector and regulatory responses in autoimmune disease, provide balance to the Th1/Th17 paradigm and help to explain the clinical heterogeneity of human uveitis, which occurs in the face of responses to the same ocular antigen(s).

Keywords T lymphocytes · T regulatory cells · Th1 · Th17 · Uveitis · Autoimmune disease

Introduction

Uveitis of a putative autoimmune nature has been estimated to affect 150,000 Americans annually [1]. Uveitis encompasses a group of potentially blinding inflammatory diseases, which may be limited to the eye, or may be associated with a systemic syndrome [2]. An autoimmune causality is supported by strong HLA associations and by frequent responses to one or more retinal antigens (Ags) [3]. Ocular trauma may precipitate uveitis, presumably through breach of the blood–ocular barrier and release of normally sequestered Ags. In most

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cases, however, the etiologic triggers are unknown and have been postulated to include antigenic mimicry by microorganisms in conjunction with a concomitant adjuvant effect, leading to the priming of effector T lymphocytes capable of recognizing ocular Ags.

A robust mouse model for human uveitis is experimental autoimmune uveitis (EAU), which can be induced by immunization of susceptible animals with a retinal Ag such as interphotoreceptor retinoid binding protein (IRBP) and retinal arrestin (retinal soluble Ag = S-Ag) [4]. IRBP is a large protein that transports retinoids, which are part of the visual cycle, between the retinal pigment epithelium and the photoreceptors. S-Ag is the visual arrestin that quenches photoactivated rhodopsin in the process of visual signal transduction. Both proteins are highly evolutionarily conserved and constitute major components of the photoreceptor cell layer. Retinal Ags that are involved in the visual cycle and that can serve as targets in EAU are typically unique to the eye. The only other site of expression (within limits of detection of currently available methods) is the pineal gland ("third eye"), which controls the circadian rhythm and shares many vision-related proteins with the retina.

The eye is an immune privileged organ and enjoys a special relationship with the immune system. Immune privilege is a complex phenomenon that involves multiple components, starting with sequestration behind an efficient blood–retina barrier, through active local inhibition by soluble and surface-bound molecules that actively inhibit activation and function of adaptive and innate immune cells, and culminating in systemic regulation via induction of T regulatory cells (Tregs) [5]. The eye is thus protected from local inflammatory responses to environmental microorganisms, which would have the potential to distort vision. However, we believe that immune privilege also leaves the eye vulnerable to autoimmune attack by lymphocytes that have been primed elsewhere in the body by chance encounter with a self or with mimic Ag(s).

It is now known that central (thymic) tolerance to retinal Ags operates similarly to central tolerance to other tissue specific Ags. Several retinal Ags, including IRBP, have been detected in the thymus and are under control of the AIRE transcription factor [6]. Different mouse strains express different amounts of IRBP in the thymus. The amount of IRBP expressed in the thymus was reported to correlate inversely with susceptibility to EAU of the strain [7]. We have shown previously that thymic expression of IRBP negatively selects T effector cells (Teffs) and positively selects natural T regulatory cells (nTregs) [8, 9]. However, negative selection in the thymus does not eliminate all autoreactive T cells [10]. Normally, autoreactive thymic emigrants that have escaped negative selection are subject to peripheral tolerance and are thus kept under control. However, since Ags resident in the healthy eye are sequestered behind a blood–retina barrier, they are not readily accessible to recirculating lymphocytes. Therefore, the process of peripheral tolerance is unlikely to operate efficiently with respect to retinal Ags. This may account for the relative ease of induction and multiple strains and species (mice, rats, guinea pigs, rabbits, monkeys) that are susceptible to EAU with one or another retinal protein [3].

The best known and best characterized model of murine EAU is induced in mice by immunization with IRBP in complete Freund's adjuvant (CFA) [4]. The pathogenic epitopes of IRBP are known for several strains of mice. The most highly susceptible strain is B10.RIII, which recognizes peptide IRBP161-180. However, a very useful strain for basic studies is C57BL/6, which is only moderately susceptible, because this is the background on which most of the knockouts and transgenics are made. The C57BL/6 strain responds to IRBP1-20. We have recently identified several additional pathogenic epitopes for each of these strains [11]. Nine to twelve days after immunization the EAU-challenged animals develop an ocular inflammation, ranging in intensity from mild inflammatory cell infiltration in the posterior pole to panuveitis and complete destruction of the retina. An

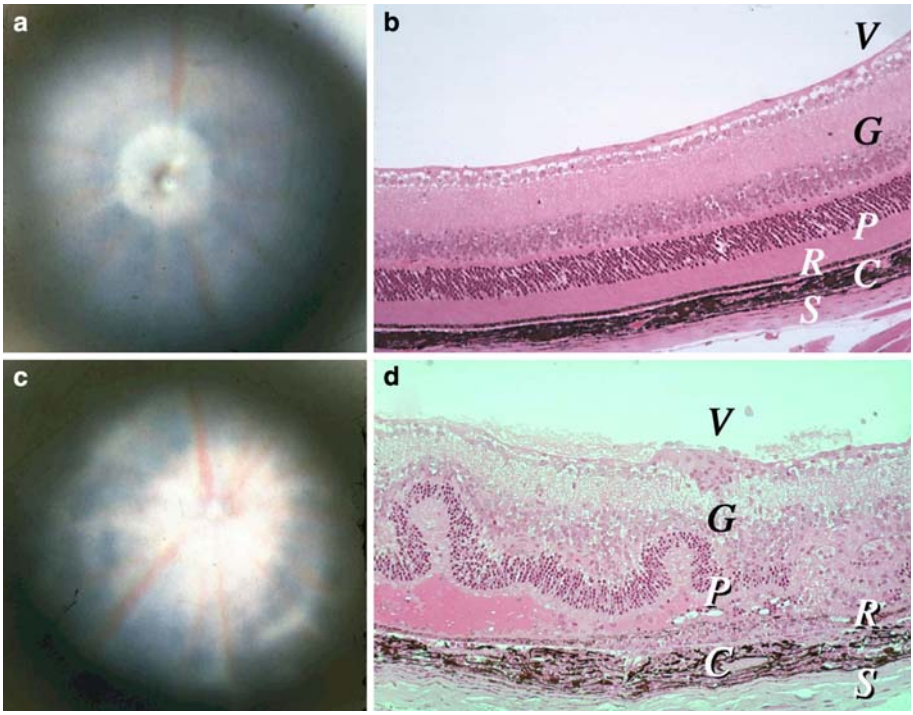

Fig. 1 Typical EAU induced with IRBP in the B10.RIII mouse. (**a** and **b**) Healthy retina by fundus examination (**a**) and by histology (**b**). Note ordered retinal layers *V*: vitreous; *G*: ganglion cell layer; *P*: photoreceptor cell layer; *R*: retinal pigment epithelium; *C*: choroid; *S*: sclera. (**c** and **d**) Uveitic retina. Note detached retina around optic nerve head, perivascular cuffing and semicircular lesions (**c**). The retinal architecture is disorganized (**d**) showing vasculitis, retinal folds, subretinal exudate, loss of nuclei in the ganglion and the photoreceptor cell layers, disruption of the retinal pigment epithelium and inflammation of the choroid. *Adapted from* [13]

alternative method of inducing disease is by infusion of lymphoid cells obtained from draining lymph nodes and spleens of immunized, genetically compatible, donors and cultured in vitro for varying periods of time with the specific Ag. The recipients will then develop typical EAU after a shortened latent period. We have shown recently that EAU can also be induced by injection of in vitro matured IRBP161-180-pulsed dendritic cells (DC) [12]. The development and severity of the disease can be followed by periodic fundus examination (fundoscopy) under a binocular microscope and by histopathology [4]. The typical appearance of EAU by fundoscopy and histopathology is shown in Fig. 1.

Figure 2 summarizes what we believe to be the important checkpoints in the pathogenesis of uveitis, starting with exposure to Ag, and culminating in ocular manifestations of the disease. This sequence is based on studies from numerous laboratories over many years. Our current studies concentrate on the generation of effector and regulatory T cells specific to ocular Ags, which impact the development of ocular autoimmunity.

Uveitogenic effector cells: Th1 or Th17?

Experimental autoimmune uveitis is a T cell mediated autoimmune disease model. It has long been known that only T cells can adoptively transfer disease, and that it cannot be

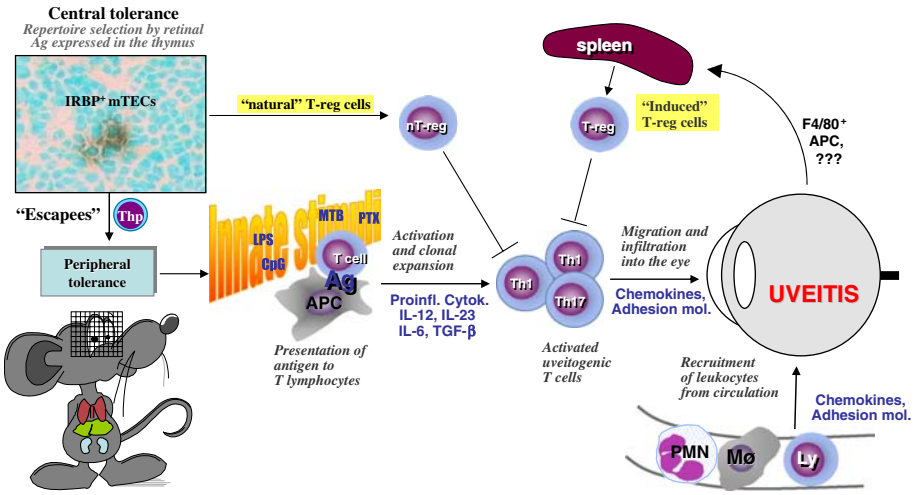

Fig. 2 Shown is a schematic representation of the checkpoints and regulatory events in the process of ocular autoimmunity. Incomplete elimination of retina-specific effector precursor T cells in the thymus, combined with deficient peripheral tolerance, leads to a circulating pool of non-tolerized T cells that can be triggered by exposure to a retina-derived or crossreactive Ag presented in the context of inflammatory danger signals. This leads to a differentiation of the activated T cells to an autoaggressive Th1 or Th17 phenotype. "Natural" Treg cells are exported from the thymus along with the effector precursors and inhibit their activation and clonal expansion in an Ag-specific as well as in a bystander fashion. Activated effector T cells migrate and extravasate at random, and some reach the eye. Recognition of the cognate Ag in the tissue is followed by downstream events culminating in a breakdown of the blood–retinal barrier, recruitment of inflammatory leukocytes, further amplification of the inflammatory process and uveitis. Retina-derived Ags released from the damaged tissue induce generation of Ag specific Tregs in a process requiring the spleen, which help establish a new type of balance and maintain functional tolerance. *Previously published in* [14]

transferred with hyperimmune serum. This, however, does not mean that Abs have no role, only that they are insufficient to initiate the disease when the blood retinal barrier is still intact. However, if a small number of T cells is transferred that by itself is insufficient to induce significant disease, serum antibodies can exacerbate EAU scores [15].

A considerable body of evidence has accumulated over the years from many laboratories that EAU is associated with a Th1 response. (i) EAU-susceptible strains tend to be high Th1 responders (high IFN-γ, low IL-4 & IL-5); (ii) long-term uveitogenic T cell lines have a Th1 phenotype; (iii) immune T cell populations that make little or no IFN-γ are non-uveitogenic, but can be converted to a uveitogenic, IFN-γ-producing phenotype by culture in the presence of IL-12 [16]. These early studies also demonstrated that IL-12p40 knockout (KO) mice and mice treated with anti-IL-12p40 mAbs are resistant to EAU, which at that time was attributed to their lack of IL-12 and inability to mount a Th1 response. However, there existed a paradox in that IFN-γ KO mice or mice treated systemically with IFN-γ neutralizing Abs actually had exacerbated disease [17, 18], putting in question the necessity of at least IFN-γ, if not of the Th1 response, as part of the effector mechanisms.

Things became clearer with the discovery of IL-23, which shares the p40 chain with IL-12, and thus neutralization or genetic deficiency of p40 results in lack of IL-23 as well. We now know that IL-23 is necessary to maintain a new effector cell lineage whose differentiation is induced by IL-6 + TGF-β, named Th17 after its signature cytokine, IL-17 [19, 20]. Studies in other autoimmune disease models, most notably experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA) demonstrated a critical role

for IL-23 rather than IL-12 in their pathogenesis, emphasizing a role for Th17 cells and de-emphasizing a role for Th1 [21, 22].

In IRBP-CFA immunized mice both Th1 and Th17 cells are induced, and both effector populations can be demonstrated in uveitic eyes by intracellular staining for the appropriate cytokine. Which effector population is, therefore, more important? In vivo neutralization of each of the hallmark cytokines, IFN-γ and IL-17, demonstrates that only neutralization of IL-17, but not of IFN-γ, is able to ameliorate EAU [17, 23]. If anything, neutralization of IFN-γ exacerbates disease [17]. IFN-γ KO mice tend to develop more severe EAU than their WT counterparts and show a high proportion of IL-17-producing CD4+ T cells in their lymph nodes and in uveitic eyes [23]. This suggested that Th17 cells may be the pathogenic effectors in IFN-γ KO mice. In keeping with this, adoptive transfer of in vitro polarized IRBP-specific Th17 cells derived from IFN-γ KO mice into unimmunized WT or IFN-γ KO hosts was able to cause EAU [23], showing that Th17 cells can be pathogenic in the absence of IFN-γ and a normal Th1 response, and supporting the notion that they can serve as standalone effectors.

IL-17 and the Th17 effector response may also have a role in human uveitic disease [24–26], although additional studies are needed to dissect this in more detail.

Despite these findings, which support an important role for the IL-23/IL-17 pathway in inflammatory autoimmunity in the eye, EAU development does not necessarily require IL-17. We found that IL-17 KO mice are not resistant to EAU [23]. Interestingly, their eyes contained a much higher proportion of CD4 cells able to produce IFN-γ than did eyes of WT controls, raising the possibility that, in the absence of IFN-γ, Th1 cells may contribute to pathology. Further, a polarized Th1 cell line unable to produce IL-17 can induce severe EAU. Treatment of WT recipients infused with this line with neutralizing anti-IL-17 Abs at a dose that prevents induction of disease by active immunization, has no inhibitory effect [23], indicating that recruitment of IL-17 producing cells from the host is not needed for pathology to develop. These findings also raise the possibility that IL-23, which—as mentioned above—is necessary for EAU development, may have additional roles in the development of autoimmunity, that are separate from its role in promoting the Th17 effector response.

We recently have developed an alternative model of EAU, induced with Ag-pulsed mature DC [12]. In this model, large numbers of DC are elicited in vivo using a hydrodynamic injection of a murine Flt3L expression plasmid. Hydrodynamic injection of DNA is a highly efficient in vivo transduction protocol. It consists of a rapid infusion through the tail vein of a large volume containing the DNA of interest. This reverses the blood flow through the inferior vena cava and delivers the DNA into the internal organs, primarily the liver, where it is expressed [27]. The Flt3L-elicited DC are purified a week later from spleens of the injected mice and are matured in vitro using LPS and anti-CD40 and pulsed with IRBP161-180. Recipients of these uveitogenic DCs develop an EAU-like ocular inflammation, which, however, differs immunologically and clinically from the "classical" EAU model induced by immunization in CFA. Its course is shorter and scores are typically milder, and the appearance of the fundus of the eye is punctate rather than continuous lesions. More importantly, the nature of the inflammatory infiltrate recruited into the eye is different: it is largely composed of neutrophils rather than the typical mononuclear infiltrate in "classical" CFA-IRBP induced EAU, indicating that the cytokines and chemokines involved in immunopathology are different. Indeed, assay of the cytokines produced by primed lymph node cells in these recipients demonstrates an IFN-γ dominated response, with relatively low production of IL-17. That this IFN-γ response has functional importance was demonstrated by infusing uveitogenic DC into IFN-γ KO mice, which failed to develop disease, although they developed a good Th17 response. This contrasts

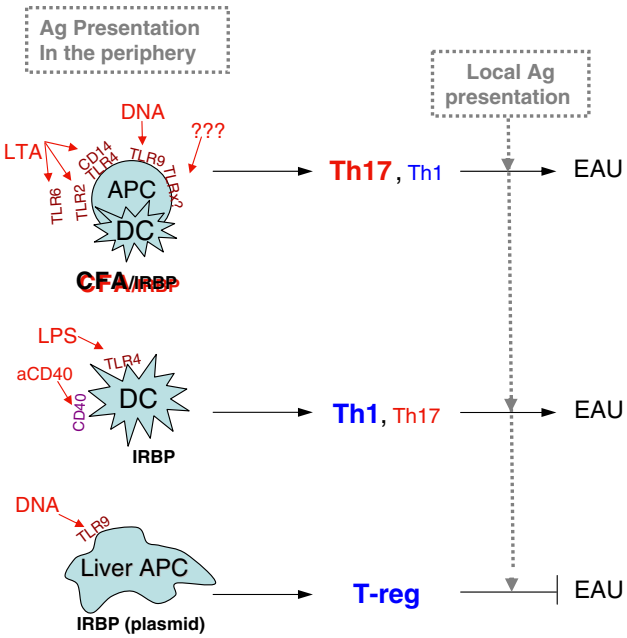

Fig. 3 Conditions of initial exposure to Ag that may determine effector dominance are the quality/quantity of TLR and other innate receptor signals and the type/variety of cells participating as APC. Not depicted is the duration of exposure to the stimulus. *Adapted and expanded from* [23]

with the exacerbated EAU developed by IFN-γ KO mice after immunization with IRBP in CFA and indicates that the DC-EAU model is functionally dependent on IFN-γ producing effector cells.

These findings demonstrate that EAU can be driven either by Th17 or by Th1 cells, depending on the model. A major difference between these two models of EAU is the context in which the uveitogenic Ag is presented to the immune system. In immunization using CFA, the Ag/adjuvant mixture reaches the draining LN, where the mycobacterial components of CFA provide strong innate stimulation to diverse antigen presenting cells over a prolonged period of time. In contrast, the Ag pulsed DC matured with only LPS and anti-CD40 constitute a much more focused and limited stimulus, resulting in a different type of response and a different type of disease (Fig. 3). These concepts may shed light on the heterogeneity of human uveitis, which is clinically diverse even though the patients may respond to the same retinal Ag(s). The nature of the initial triggering stimulus in human disease is of course largely unknown. The implications for therapy are that in some uveitic diseases the IL-23/IL-17 pathway may be a good target for therapy, whereas in other types of uveitis targeting the Th1 pathway may be more appropriate.

Innate production of Il-17 and IFN-γ: possible regulatory role in uveitis

Emerging data from different labs demonstrates that IL-17 is produced not only by adaptive, but also by innate immune cells. We found that IL-17 is produced by NKT cells. Unlike the adaptive IL-17 response, which takes several days to develop, the innate IL-17

secretion by NKT cells occurs literally within hours and is IL-6 independent, due to constitutive expression by NKT cells of IL-23R and the Th17-specific transcription factor, RORγt (which in adaptive Th17 cells are upregulated by IL-6) [28]. Although all types of NKT cells (type I, type II, and type III, as classified by their T cell receptor (TCR) and CD1d dependence) produce IL-17, it is produced only by the relatively small NK1.1-negative fraction. As a consequence, its systemic production after in vivo administration of the NKT-specific ligand α-GalCer is low and cannot be detected in the serum. In contrast, IFN-γ is produced by NK1.1-positive as well as -negative NKT cells, in quantities easily detectable in serum after in vivo administration of α-GalCer [28, 29].

Although the influence of innate IL-17 production on development of EAU remains to be determined, innate IFN-γ induction from NKT cells at the time of uveitogenic challenge can regulate EAU development. Treatment of IRBP-CFA immunized mice with α-GalCer analogs that trigger different amounts of IFN-γ from NKT cells inhibits EAU, and the most protective analog is the one that causes highest production of IFN-γ [29]. The protected mice exhibit reduced adaptive IL-17 and IFN-γ responses to IRBP. Concurrent treatment with a neutralizing IFN-γ Ab reverses the protection and restores adaptive IFN-γ and IL-17 responses. Thus, unlike locally produced IFN-γ, which enhances pathology by inducing a broad spectrum of proinflammatory mediators, elevated systemic levels of IFN-γ at the time of priming restrain development of adaptive immunity. These data help to reconcile the apparent paradox that genetic lack or systemic neutralization of IFN-γ results in exacerbated EAU scores in the IRBP-CFA model. They are also in line with our earlier findings that in vivo neutralization of IFN-γ can counteract genetic resistance to EAU in some strains, and that upregulation of systemic IFN-γ in susceptible mice at the time of priming (by infusion of IL-12) protects from EAU [17, 30].

Natural and induced regulatory cells in uveitis

As an immune privileged organ, the eye is capable of inducing complex regulatory circuits that culminate in induction of CD4+ and CD8+ regulatory cells. However, these become apparent after the eye sustains some kind of damage, whether through injection of an antigen into the eye or after recovery from EAU [31, 32]. These induced Tregs might be able to ameliorate re-induction of disease, but they would not be present in a naïve animal to protect from an initial exposure to a uveitogenic stimulus.

Thymic-derived natural T regulatory cells (nTregs) are positively selected in the thymus on ectopically expressed tissue Ags and restrain autoimmune responses to a variety of tissues [33]. The knowledge that the thymus expresses retina-specific Ags prompted us to investigate whether nTregs might also control responsiveness to retinal Ags. Indeed, depletion of CD25+ cells before uveitogenic immunization greatly exacerbated EAU scores and associated adaptive immunological responses and even permitted EAU to develop in resistant BALB/c mice, demonstrating that nTregs raise the threshold of susceptibility to EAU [9 and unpublished data]. The generation of these nTregs is dependent on expression of IRBP, as they are not present in IRBP$^{-/-}$ mice. Surprisingly, however, generation of IRBP-specific effector cells and induction of EAU can be inhibited also by CD25+ Tregs of unrelated specificities that are activated by mycobacterial components present in CFA [9]. We hypothesize that stimulation of Tregs by mycobacterial components occurs through innate immunity receptors, either directly or indirectly. The finding that Tregs of unrelated specificities triggered by CFA can dampen autoimmune disease may relate to the “hygiene hypothesis”. This hypothesis seeks to explain the observation that in undeveloped

countries, where exposure to microorganisms is more frequent, there are less allergies and autoimmune diseases than in the developed world. It is conceivable that chronic stimulation of nTregs by microbial components might serve to raise the overall threshold of immune responsiveness, thereby limiting responses to autoantigens and allergens.

Although it is clear that nTreg cells have an important role in setting the threshold of susceptibility to EAU, in susceptible individuals they are insufficient to prevent disease, or have already failed. Reduced Treg function has been reported in uveitis patients [34]. These concepts support a rationale to study the feasibility of inducing Tregs as a therapeutic approach. A number of strategies have been shown to promote generation of Treg cells, among them: IV antigen, altered peptide ligands, mucosal administration of Ag and tolerogenic DNA vaccination. We chose the latter approach.

As our vaccination method we used hydrodynamic intravenous injection (as described above for Flt3L) of a DNA plasmid encoding the first homologous repeat of IRBP, which contains the IRBP161-180 epitope. IRBP could be detected in the liver by Western blotting within 8 h of vaccination. Vaccinated mice were highly protected from EAU induced by immunization with IRBP and were significantly protected also in a reversal protocol, indicating the utility of this approach in ongoing disease [35]. Interestingly, although detectable IRBP signal in the liver was gone by 50 h after vaccination, the vaccinated animals remained highly protected from a uveitogenic challenge for at least 10 weeks. The mechanism of protection appeared to involve primarily CD4+ CD25+ Treg cells that could be expanded in culture using IRBP161-180-pulsed bone marrow DC, and that transferred protection to naive recipients. In vitro characterization of these cells revealed that they are Ag specific, anergic, express FoxP3, CTLA-4 and GITR, and suppress by contact.

Thus, expression of IRBP outside of the eye by DNA vaccination constitutes a tolerogenic exposure to Ag, which results in generation of IRBP specific regulatory T cells, whose effector function is to inhibit other T cells. DNA is a TLR9 stimulant. The APC are involved in this tolerogenic presentation remain to be characterized, but since most of the expression is in the liver, these are likely to be liver-associated APC (Fig. 3). Treg cells may offer a new approach to Ag-specific therapy of uveitis and the laboratory is continuing to explore and characterize their role in controlling autoimmunity in the eye.

Other ongoing and future research directions

The laboratory has a number of other ongoing research activities. Some are a direct extension of questions raised by the studies described above, and some constitute separate research directions. These include:

- Genetic susceptibility to EAU: in vivo and in silico studies using classical genetic, genomic, and bioinformatic approaches [36, 37].
- Characterization of HLA transgenic (Tg) mice and their responses to retinal Ag, as an approach to dissecting the uveitogenic epitopes that may be relevant to human uveitis [15].
- Development and characterization of IRBP TCR Tg mice—a spontaneous model of EAU.
- The role in autoimmunity of innate IL-17 produced by NKT cells and $\gamma\delta$ T cells.
- Dissecting the innate stimuli that drive towards a Th17 or Th1 dominated response in vivo.
- The role of IL-22 in ocular inflammation. This Th17-associated cytokine has opposing effects in different tissues, from proinflammatory (psoriasis) to protective (hepatitis) [38, 39]. It is expressed in uveitic eyes, but its role in ocular pathology is unknown.

- Further insights into ocular immune privilege: characterization of the inhibitory function of retinal glial Müller cells, which are able to inhibit the proliferation and function of uveitogenic Teff cells [40]. We also study the possible role of retinoids in the eye, which are important in the visual process, in inducing eye-related Tregs.
- The mechanism of non-specific activation of Tregs by microbial components.
- Characterization of the in vivo mode of action of Treg cells, including migration patterns, site of action and their effects on Teff cell function.

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